EXHIBIT G

ELSEVIER

Vaccine 17 (1999) 1596–1604

Vaccine C5

New technologies for making vaccines

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Abstract

Technologies for making active vaccines fall into 3 general groups: live, subunit (killed or inactivated) and genetic. Each of these groups is further divisible into multiple categories, which include recombinant-derived antigens as well as native microorganisms and their components. In addition, there are new enabling technologies such as delivery systems and vectors which can be applied to these approaches. Most disease targets, whether infectious or noninfectious in origin, can be approached by the application of several different vaccine technologies, as can be tested during the discovery phase of research. The criteria for choosing early in a development program which of the vaccine technologies are likely to ultimately be most fruitful for a given application include: knowledge of the pathogenesis of the given infection/disease; technical feasibility; immunobiology and associated mechanisms; preclinical efficacy profile; anticipated clinical safety; regulatory; manufacturing; and marketing. All of these criteria should be considered together in making selections for an R&D program. This paper is reviewing the major vaccine technologies and relevant examples of how these criteria are used to make decisions in vaccine development.

1. Introduction

The past 2 decades have witnessed a huge increase in the number of technological and immunological approaches for making new vaccines. This chapter will discuss the major technologies, immunological objectives and key issues for making different kinds of active vaccines. These discussions and the individual examples presented should provide the reader with a strong framework for appreciating the diverse approaches being taken to the research and development of new vaccines.

There are three general categories of active vaccines, whose salient features are outlined in Table 1. The strategic decision for developing a live, subunit or genetic vaccine is best made after considering the pathogenesis, epidemiology and immunobiology of the infection or disease in question as well as the technical feasibility of each approach. Epidemiology dictates the target population for the vaccine. The age and state of health of this population may favor some strategies as more appropriate for eliciting protective immunity. For example, minimal reactogenicity is very important for a vaccine intended for healthy infants, while the degree of reactogenicity is less important in cases such as a therapeutic cancer vaccine. A knowledge of immunobiology can aid in the identification of the type of protective immunity that should be elicited by the vaccine; certain immune responses may be protective and others useless, or even detrimental, to prevention or treatment. Clearance of the natural infection may correlate with the appearance of antibodies against a particular antigen, thereby defining that antigen as an immunogen for a candidate vaccine. Historically, only some technical approaches have been judged to be feasible for a particular vaccine. Nevertheless, considering the expanding number of technical approaches, it may become possible to custom-design many vaccines for optimal efficacy and tolerability. Also, while all currently-licensed vaccines are prophylactic, there are many therapeutic vaccines in clinical trials.

2. Live vaccines

Some live vaccines come very close to meeting the criteria for an ideal vaccine of being able to elicit lifelong protection with minimal reactogenicity after only one or two doses. Such vaccines consist of microorganisms (usually viruses) that replicate similarly to the natural microorganism in vivo, thereby eliciting an immune response similar to that elicited by the natural

0264-410X/99/$19.00
PII: S0264-410X(98)00416-2

Table 1
Comparative properties of active vaccines

Live vaccines
● Able to replicate in the host
● Attenuated in pathogenicity
Advantages:
May elicit broader immune responses
May require fewer doses
Generally longer lasting protection
Subunit vaccines (killed inactivated)
● Unable to replicate in the host
Advantages:
Cannot multiply or revert to pathogenicity
Generally less reactogenic
Nontransmissible to another person
Usually more feasible technically
Genetic vaccines (DNA-based)
● Stimulate synthesis of antigens only in cells
Advantages:
Elicit cellular immune responses
Standardized method of production

infection. The live vaccine is attenuated by technical or biological manipulations for eliminating its disease-causing capacity. The live vaccine should be neither overattenuated, such that it no longer replicates sufficiently to function as a vaccine, nor underattenuated, whereby it retains even limited pathogenicity or ability to revert to pathogenicity. Live vaccines usually elicit both humoral immunity (antibodies) as well as cellular immunity [e.g. cytotoxic T-lymphocytes (CTL)]. Some live vaccine strains can be transmitted from the vaccinee to an unvaccinated individual, which can be quite serious if the recipient is undergoing cancer chemotherapy or has an immunodeficiency disorder. The natural viral infection per se may fail to produce a protective immune response, such that an attenuated virus (without further molecular engineering) ordinarily would not be expected to produce a protective response.

2.1. Classical strategies; viral

The term *classical* refers to technical strategies that do not utilize rDNA technology.

2.1.1. Attenuation in cell culture

The wild-type virus isolated from a natural human infection is passaged in vitro through one or more cell types in order to attenuate its pathogenicity. In some cases (e.g. poliovirus [1]), it has been possible to demonstrate attenuation in a primate species, whereas in most cases attenuation can be proven only after extensive clinical trials. This empirical approach has been successful for both an oral vaccine (oral poliovirus vaccine (OPV) [1]) and injected vaccines (measles, mumps, rubella [2]). The reactogenicity of such vaccines has been low enough that some of them (polio, measles) are widely accepted worldwide for routine pediatric use. By means of intensive immunization with OPV, polio is well on its way to worldwide eradication.

2.1.2. Temperature-sensitive mutants

Temperature-sensitive mutants can be selected according to their growth properties at different temperatures. These viruses have been referred to as temperature-sensitive (ts), being unable to grow at elevated temperatures, or cold-adapted (ca), having been selected for growth in vitro at lower than physiological (37°C) temperatures, i.e. down to 25°C. The intention is that the ca or ts viruses will replicate less vigorously in vivo than their respective wild-type parental viruses, hence be phenotypically attenuated and less virulent. A ca influenza vaccine has been tested widely [3], and a double ts respiratory syncytial virus (RSV) vaccine has been tested clinically with some promise [4]. The use of this double ts mutant is a refinement resulting in a much lower frequency of reversion to wild-type virulence than for a single ts mutant.

2.1.3. Variant viruses from other species

Variant viruses from other species that cause a disease similar to a human disease may be cultivated with the goal that the animal virus will become attenuated for humans yet be sufficiently related immunologically to the natural human virus for eliciting protective immunity to the human agent. The worldwide immunization program using vaccinia virus [5], the prototype variant virus, resulted in the complete eradication of smallpox by the mid-1970s, the only infectious disease ever eradicated. Based on this model, first-generation vaccines for rotavirus consisted of animal-derived viruses [6]; however, these rotavirus vaccines were not reproducibly efficacious in humans.

2.1.4. Reassortant virus

A reassortant virus is derived following coinfection with two different viruses with segmented genomes. Reassortant rotaviruses were isolated containing mostly animal rotavirus genes, which confer the attenuation phenotype for humans, as well as a single gene from a human rotavirus surface protein which elicits serotype-specific neutralizing antibodies for human rotavirus [7]. These reassortant rotaviruses have elicited higher efficacy rates as vaccine candidates than their nonreassortant parental animal viruses. One such quadrivalent vaccine has been licensed in the US.

2.2. Recombinant virus

Specific modifications or deletions can be made in viral genes so that the virus is more stably attenuated, i.e. highly unlikely or unable to revert to virulence. In contrast, attenuated viruses derived by classical strategies may have only point mutations and therefore be able to revert. Deletions have been introduced into herpes simplex virus (HSV) which make it unable to replicate. This recombinant virus is produced in vitro in a cell line which supplies the deleted gene in *trans* and the resultant virus can initiate infection in vivo without being able to replicate further [8].

2.3. Recombinant viral vector

The second application of rDNA technology to the development of new live vaccines has been the engineering of viruses (or bacteria) as carriers or vectors of foreign recombinant polypeptides or epitopes from other pathogens. The strategy is to present the recombinant antigen in the context of a live virus infection so that the immune system responds to that antigen as a *live* immunogen and thereby develops broader immunity (humoral and cellular) to the corresponding human pathogen. The recombinant polypeptide is expressed within the virus-infected cell and (i) is transported to the cell surface or secreted in order to stimulate antibody production or (ii) is broken down into peptide fragments that are transported to the cell surface where they elicit CTL responses. The immunogenic signal may be amplified when the live vector initiates multiple rounds of replication. However, the nature of the immune response to the live viral vector per se may limit the effectiveness of revaccination.

The prototype viral vector is vaccinia virus. An expression cassette, with the foreign gene of interest flanked by vaccinia viral sequences, is taken up simultaneously with vaccinia virus into the cytoplasm where they undergo a homologous recombinational event, thereby producing a recombinant vaccinia virus expressing the foreign polypeptide. Recombinant vaccinia virus expressing the major envelope glycoprotein (gp120) of human immunodeficiency virus type 1 (HIV-1) has been tested clinically [9] for prophylactic and therapeutic applications. In order to selectively manipulate the immune response to a vaccine antigen, a recombinant poxvirus vector has been constructed which expresses both a cytokine as well as the foreign antigen [10]. Adenovirus strains, which have been used extensively as vaccines in military recruits to prevent acute respiratory disease, also have been engineered to express foreign polypeptides and have elicited protective immunity in several viral challenge models in animals [11].

RNA viruses can be engineered similarly. Sindbis and other alphaviruses have received attention due to their broad host range, ability to infect nondividing cells and potential high level of expression [12].

2.4. Classical strategies: bacterial

The one widely available live bacterial vaccine based on serial in vitro passage is for tuberculosis. A strain of *Mycobacterium bovis*, bacille Calmette-Guérin (BCG) [13], was attenuated by 231 successive in vitro subculturings over 13 years. There are many strains of BCG vaccine available worldwide that were derived from the original strain isolated in the early 20th century. These vaccines vary in terms of tolerability, immunogenicity and rate of protective efficacy in clinical trials (range of 0–80% protection) for reasons which may relate to the actual vaccine strains used or to differences in study populations.

The Ty21a strain of *Salmonella typhi* was derived chemical by mutagenesis followed in vitro selection [14]. It was licensed for the prevention of typhoid fever based on its record of safety and efficacy after a regimen of 4 doses [15]. Given that techniques of rDNA technology now can be applied to attenuation of a new bacterial strain, it seems unlikely that a new live bacterial vaccine attenuated by a classical strategy alone will be developed.

2.5. Recombinant bacteria

The strategy is to identify the gene(s) responsible for the virulence of the bacteria and to either eliminate the gene (preferred) or to abolish its in vivo expression. As for mutant viruses, there can be a balance between the virulence of a bacterial strain and its activity as a vaccine. *Vibrio cholerae* strains have been attenuated by the rDNA-directed deletion of genes that encode virulence factors (such as cholera toxin (CT), which is encoded within the *CTX* genetic element) [16]. Live attenuated cholera vaccine candidates prepared in this fashion have been evaluated clinically and one licensed. In order to assure attenuation by reducing the probability of reversion, it is desirable to delete two or more independent genes or genetic loci which contribute to virulence.

2.6. Recombinant bacterial vector

Pathogenic bacteria can be attenuated and engineered into live recombinant vectors for expressing foreign polypeptides encoded by other microorganisms. The most common applications have been for enteric bacteria which can induce mucosal immunity against the foreign polypeptide upon oral delivery. The ability

of some of these bacterial species to replicate intracellularly may augment the ability of expressed foreign polypeptides to elicit cellular immune responses against their respective pathogens. *S. typhi* has been the focus of the most effort in the field in terms of strain development, immunology and clinical evaluations [17]. The BCG vaccine strain also has been engineered as a live vector to express foreign genes, including several from HIV-1 [18].

3. Subunit vaccines

Nonlive vaccines do have certain advantages that relate to their inability to multiply within the host (Table 1). Generally they are well tolerated, especially those that undergo purification to remove other macromolecules. The immunogenicity of a nonlive vaccine usually is enhanced by an adjuvant or delivery system [19]. Any nonlive vaccine should be developed with the realization that multiple doses, often followed by booster doses, usually are necessary for attaining long-term protective immunity. In some exceptional cases, short-term protection has been demonstrated following a single dose [20]. Nonlive vaccines usually function by stimulating humoral immune responses as well as by priming for immunological memory. In certain cases, especially when administered with certain adjuvants and delivery systems, nonlive vaccines may stimulate CTL immunity.

3.1. Whole pathogen

The earliest approaches to making nonlive vaccines relied on inactivating whole bacteria or viruses in order to elicit neutralizing antibodies to directed against one or more antigens.

3.1.1. Inactivated bacterial vaccines

Inactivated bacterial vaccines are prepared by cultivating the bacteria, e.g. *Bordetella pertussis* (P), collecting the whole bacterial cells and inactivating them with heat or with chemical agents such as thimerosal or phenol [21]. The final vaccine does not undergo further purification. Owing to their biochemically highly crude nature, which includes all bacterial cellular components, the reactogenicity of such whole-cell P (P_w) vaccines when given parenterally is usually greater than that of other types of vaccines. On the other hand, inactivated whole-cell *V. cholerae* vaccine [22] has been well-tolerated by the oral route. Given the many alternative technologies available for preparing purified vaccines and the current more exacting regulatory standards, relatively few new killed whole-cell bacterial vaccines are likely to become available in the future.

3.1.2. Inactivated viral vaccines

Inactivated viral vaccines are generally very well tolerated. Cell-free media from virus-infected cultures are collected (e.g. poliovirus [23] and influenza virus [24]); the large size of the virus particles relative to other macromolecules in the media enables the particles to be enriched readily by simple purification techniques. The virus particles are inactivated chemically, typically by treatment with formalin, and then adjuvanted by an aluminum salt. The key surface epitope(s) of many nonenveloped small viruses that elicits a protective immune response (protective epitope) is usually conformational, being formed by the highly-ordered assembly of structural proteins into precise structures. For most such viruses, it may not be possible to mimic the conformation of such epitopes by other technologies, e.g. recombinant polypeptides. Inactivated viral vaccines tend to be highly potent immunologically, one of which has demonstrated protective efficacy following a single dose of vaccine [20]. Thus, this classical strategy, which has had an excellent track record of producing well-tolerated and efficacious vaccines, has remained the technology of choice for many viral vaccines.

3.2. Protein-based

Developing a (purified) protein-based vaccine is the strategy of choice for many pathogens in which a polypeptide contains protective epitopes and assuming that an inactivated vaccine is technically not feasible or undesirable. In addition to traditional ways of discovering vaccine antigens, there are newer enabling technologies such as genomics which also are being employed to identify novel antigens.

3.2.1. Natural

The first protein-based vaccines relied on natural (nonrecombinant) sources of antigens. Liver cells of individuals chronically infected with hepatitis B virus (HBV) shed excess viral surface protein, i.e. hepatitis B surface antigen (HBsAg), into blood. Plasma was harvested from chronic carriers of hepatitis B, HBsAg purified, and the final preparation subjected to 1–3 inactivation techniques (depending on the manufacturer) to kill HBV and any other infectious agent that might have been present in the starting plasma [25]. This highly efficacious vaccine is unique among active vaccines in that it utilized a human tissue source (plasma) for the vaccine antigen.

Proteins purified from cultures of *B. pertussis* can be combined to formulate acellular pertussis (P_a) vaccines, which eventually may replace P_w vaccines for routine pediatric vaccinations in many developed countries. Depending on the number of different protein antigens, these P_a vaccines are referred to as one-, two-, three-, four-, or five-component vaccines and have

been licensed based on recent efficacy studies [26]. These vaccines all contain pertussis toxoid (PT) as a component (see below).

3.2.2. Chemical inactivation

Many bacteria produce toxins that are responsible for the pathogenesis of infection. It had been recognized for many decades that, when a toxin was the chief mechanism of pathogenesis after infection by the causative bacteria, neutralizing toxin activity in vivo could prevent or ameliorate symptoms of some bacterial infections. This precedent established the basis for bacterial toxins to be formulated as active vaccines. The toxin molecules are purified from bacterial cultures, e.g. *B. pertussis*, *Clostridium tetani* (T) and *Corynebacterium diphtheriae* (D) and then detoxified by incubation with a chemical such as formalin or glutaraldehyde. Such inactivated toxins (*toxoids*) represent two of the vaccines in the diphtheria, tetanus and pertussis (DTP) combination vaccine [27,28].

3.2.3. Genetic inactivation

rDNA technology has been employed to produce a more stable and potent toxoid. As applied to pertussis vaccine, the toxin gene was cloned and sequenced and codons for amino acids required for toxin bioactivity (adenosine diphosphate (ADP) ribosyltransferase) were mutated. The altered gene was substituted for the native gene in *B. pertussis*, which then produces immunogenic but stably inactivated PT. As a refinement of this strategy, two mutations were introduced into PT to ensure the inability to revert [29]; this double-mutant PT (which also is treated with formalin under mild conditions to improve its immunogenicity or stability) is a component of a P_a vaccine [26].

3.2.4. Recombinant polypeptides

The first application of rDNA technology to the production of a vaccine was for hepatitis B. Expression of the HBsAg gene in bakers' yeast *S. cerevisiae* [30] resulted in the expression of 22-nm HBsAg particles within cells. HBsAg is a virus-like particle (VLP) in that its surface is similar to that of HBV virions. The yeast-derived vaccine, which has been available worldwide since the late 1980s, has largely supplanted the equally-efficacious plasma-derived vaccine.

Particles can elicit antibodies which recognize conformational epitopes on the particle, while isolated surface polypeptides of the particle might not elicit the production of such antibodies. Examples of such particle immunogens are HAV particles [20] (which are immunogenic in humans at dosage levels as low as 50 ng) and HBsAg. Another recent example of the effective use of VLPs is for human papilloma virus (HPV), currently in clinical trials. The HPV virion is a highly ordered structure whose major protein is L1. *E. coli*-expressed L1 is a polypeptide which, after immunization, elicits anti-L1 antibodies which do not bind to papillomavirus virions or VLPs. However, the expression of L1 in eukaryotic cells, e.g. *S. cerevisiae*, results in the formation of L1 VLPs which after immunization elicit neutralizing antibodies which bind to VLPs and to virions [31].

Especially noteworthy are recent vaccine applications outside the realm of infectious diseases, including autoimmunity, fertility, allergy and cancer. During the period before the development of clinical type-1 diabetes, autoantibodies become detectable to pancreatic β-cell autoantigens (e.g. insulin), following which destruction of β cells ensues. A small clinical trial showed that the subcutaneous injection of recombinant *E. coli*-derived insulin into pre-diabetic patients with detectable anti-β-cell autoantibodies resulted in a significant delay in the development of clinical type-1 diabetes [32].

3.2.5. Carrier

Yeast Ty is a particle assembled in *S. cerevisiae* that cannot replicate in mammals. The foreign polypeptide can be expressed with Ty as mixed particles [33]. Being expressed on the surface of these large particles, the immunogenicity of foreign proteins might be enhanced.

3.3. Peptide-based

In many cases, it has been possible to map B-cell epitopes against which neutralizing antibodies are directed or to map T-cell epitopes for eliciting CTL activity. T-cell epitopes and some B-cell epitopes may be fully antigenic as short linear sequences in the range of 6–20 consecutive amino acid residues.

3.3.1. Fusion protein for B-cell epitopes

The immunogenicity of linear B-cell epitopes can be increased by making a genetic fusion of defined epitopes to a carrier protein that forms a large particle. A commonly used protein fusion partner of this type is HBsAg [34]. The fusion can be at the N-terminus, the C-terminus, or the internal portion of the polypeptide sequence of the protein partner, depending on which location affords the best immunogenic presentation while maintaining efficient particle formation.

3.3.2. Conjugated B-cell epitopes

The peptide can be conjugated to a carrier protein through covalent chemical linkage. The most commonly used carrier proteins in conjugates are bacterial proteins that humans commonly encounter such as T toxoid, for which a conjugate with the malarial CS epitope has been tested clinically [35].

3.3.3. T-cell epitopes

Peptide epitopes recognized by CTL may be useful immunogens for the prophylaxis of infections by agents such as HIV and *Mycobacterium tuberculosis* or immunotherapy for chronic diseases. CTL epitopes generally are not good immunogens as peptides per se. Thus, for an immunotherapeutic hepatitis B vaccine, a CTL epitope from the HBV core protein was modified by covalent linkage to a T-helper epitope (from T toxoid) as well as two palmitic acid molecules [36]. This vaccine was shown in clinical studies to be immunogenic in a 500-μg dose for eliciting HBV-specific memory. In contrast, a peptide representing a CTL epitope from the *ras* oncoprotein is immunogenic on its own in eliciting specific CTL activity in individuals with tumors expressing the *ras* oncoprotein [37].

3.4. Polysaccharide-based

There are many bacteria with an outer polysaccharide (Ps) capsule. In many if not most of the encapsulated bacteria studied, antibodies directed against capsular Ps are protective against infection. These observations have established capsular Ps as vaccine antigens.

3.4.1. Plain Ps

The natural capsular Ps contains up to hundreds of chemically-defined repeat units distinct for each bacterial species and antigenic subtype, in which each monomer consists of a combination of monosaccharides, small organic moieties and phosphate groups. The Ps is harvested and enriched from the culture medium. These Ps preparations are usually immunogenic in adults and children over 2 years of age. The Ps elicits antibodies which may mediate the opsonization of the organism, thereby protecting against bacterial infection. Ps vaccines have been licensed for *Neisseria meningitidis* [38] (quadrivalent) and *Streptococcus pneumoniae* (23-valent) [39]. The shortcomings of these vaccines is that the Ps, being T-cell-independent (TI) immunogens, are poorly immunogenic or nonimmunogenic in children younger than 2 years owing to the immature status of their immune systems and they do not elicit immunological memory in older children and adults.

3.4.2. Conjugate

Although infants and children younger than 2 years old do not recognize TI immunogens efficiently, they can recognize and respond immunologically to T-cell-dependent (TD) immunogens such as proteins. The chemical conjugation of Ps to a carrier protein converts the Ps from a TI to a TD immunogen. Thus, Ps-protein conjugate vaccines can elicit protective IgG and immunological memory in infants and young children. This strategy is particularly important for invasive diseases caused by encapsulated bacteria such as Hib and *S. pneumoniae* (pneumococcal; Pn) in children younger than 2 years old, in whom a Ps vaccine is ineffective. There are four different licensed Hib conjugate vaccines [40], all with different carrier proteins (T or D toxoids, mutant D toxoid and an outer membrane protein complex from *N. meningitidis* type B) of different sizes and immunological character, distinct Ps chain lengths and distinct conjugation chemistries. Given these differences, these conjugate vaccines display several differences in their immunological properties in infants.

The Hib bacteria are a single serotype ('*b*'). Pn bacteria, on the other hand, consist of over 80 serotypes, as reflected in distinct capsular Ps structures. For designing a pediatric Pn conjugate vaccine, 8 serotypes have been recognized as responsible for 70–90% of the major pediatric Pn diseases (acute otitis media, pneumonia, meningitis). Therefore, vaccines being tested in advanced clinical trials consist of a mixture of individual Pn Ps conjugates [41].

3.5. Anti-idiotypic antibodies

The *idiotype* (*Id*), i.e. idiotypic determinant, is associated with the hypervariable region of the antibody molecule and represents its unique antigenic determinants. An antibody (Ab-1) can be defined as recognizing a candidate vaccine antigen. The *Id* on Ab-1 itself can act as an antigen and elicit an immune response; the antibodies that bind to the *Id* on Ab-1 are referred to as *anti-idiotypic antibodies* (anti-Id) or Ab-2. The *paratope* is the site on Ab-1 that binds to the particular antigen; thus, the antigen binding site of an anti-*paratope* antibody (anti-*Id*) is a molecular 'mimic' of the antigen. By virtue of the antibody-binding site of Ab-2 mimicking the conformation of a candidate vaccine antigen, Ab-2 molecules themselves can be nonlive vaccine candidates in which an epitope (mimicked by the anti-*Id* on Ab-2) is presented on a carrier molecule (the whole Ab-2). A demonstration that deriving anti-*Id* represents a vaccine strategy comes from the demonstration that vaccination of chimpanzees with anti-*Id* that mimicked HBsAg protected the animals from infection with a pathogenic dose of HBV [42]. Certain tumor antigens cannot be recognized immunologically by the host because these antigens are self-antigens, often being expressed in low levels in the host. Nevertheless, the Ab-2 that is the mimic of the tumor antigen, yet not necessarily identical in structure to the antigen, can elicit an immune response against the tumor antigen [43]. The ultimate utility of anti-*Id* as a vaccine strategy remains to be established and the situations in which the use of anti-*Id* would be the preferred vaccine strategy are quite limited in number.

Furthermore, to obtain the most specificity as a vaccine candidate, one would derive a monoclonal antibody as an anti-*Id* and make it into a recombinant human or humanized MAb.

4. Genetic

A recent novel approach has been the use of DNA encoding a vaccine antigen (Table 1). The in vitro paradigm for this approach lay in the transformation of mammalian cells in culture with a plasmid that directs the synthesis and secretion of a vaccine antigen from cells that take up the plasmid DNA. After cells in vivo take up DNA encoding vaccine antigen(s), the antigens can be secreted or can be associated with the cell surface in a way that would trigger a humoral or cellular immune response. Furthermore, the uptake of DNA can be facilitated by chemical formulation or delivery by a virus or bacteria.

4.1. Purified DNA

One strategy has been to inject intramuscularly a solution of uncoated or 'naked' DNA encoding a vaccine antigen [44]. Cells take up the DNA, transcribe its expression cassette and synthesize the antigen, which may be processed in a similar way as in a live viral infection. Humoral or cellular immune responses to the encoded antigen are elicited. The advantages of using DNA are the relative technical ease of preparation and the ability to direct the synthesis of multiple copies of mRNA, hence an expected amplification of both antigen synthesis and immune response. Such vaccines have been shown to be effective in many animal models of infection. DNA vaccines are particularly proficient at eliciting cellular immune responses. Nevertheless, clinical efficacy for such vaccines remains to be shown.

A variation on the design of the expression plasmid is to use a virus-based DNA expression system which can amplify the level of RNA and protein expression as occurs in a live virus infection; such a system has been developed based on Sindbis virus DNA vectors [45].

Facilitation can be at the level of cellular uptake, expression or immunological activation. One strategy has been the presentation of DNA on gold microprojectiles that then are 'shot' directly into cells, which produce the encoded antigen that stimulates an immune response [46]. DNA also has been coated with cationic lipids, lipospermines or other molecules which neutralize its charge and have lipid groups for facilitating cellular uptake and membrane transfer [47]. Such formulations also are being researched for routes of injection other than parenteral which may elicit mucosal immunity. The anesthetic bupivacaine given in conjunction with DNA has been shown to enhance DNA uptake and expression [48] The base composition of the DNA may affect its potency in that unmethylated CpG dinucleotides have been shown to induce B-cell proliferation and immunoglobulin secretion [49].

4.2. Viral delivery

For delivery of DNA by fowlpox or canarypox virus, the expression cassette for the recombinant protein is integrated into the viral genome. Although able to infect avian species and produce infectious virus, these avian poxviruses can infect mammalian (human) cells but not produce infectious virus [50]; hence this can be considered a genetic vaccine approach. This single round of self-limiting infection may be sufficient to elicit broad immunity to a pathogen whose recombinant polypeptide is expressed by these avian poxviruses in infected cells, while at the same time reactogenicity should be less than that associated with vaccinia virus given the inability of the virus to spread within the host.

4.3. Bacterial delivery

Bacteria which replicate intracellularly can be engineered to deliver plasmid DNA into cells for the expression of recombinant proteins [51]. *S. flexneri* has been attenuated by making a deletion mutant in the essential *asd* gene. While such an attenuated strain can be propagated in vitro in the presence of diaminopimelic acid (DAP) and can invade cells (as long as it maintains a plasmid encoding invasion-associated polypeptides), it cannot replicate in vivo, where DAP is unavailable. A plasmid harboring a eukaryotic promoter and recombinant gene was transformed into this strain. The resultant recombinant *S. flexneri* strain was shown to be able to invade mammalian cells in vitro and to express the plasmid-encoded protein as a potential vaccine antigen. Since *S. flexneri* replicates in the intestine and stimulates mucosal immunity, this vector may be delivered orally and be effective for delivering DNA to cells where mucosal immunity is stimulated.

5. Combined approach

There are cases where a vaccine can be made by combining components made by different technologies. Oral inactivated whole-cell cholera (WCC) vaccine, which lacks CT (and its associated toxicity), has been shown to be very well tolerated and to have a rate of efficacy of ca. 60% for 3 years in a high-risk population [22]. In order to elicit antibodies that would neutralize CT, the recombinant B subunit of

CT (rCTB, which lacks toxin activity) is independently expressed, purified and added back to the WCC vaccine. This combined WCC + rCTB vaccine has been shown to have a somewhat higher rate of efficacy than WCC alone [52] Moreover, since CTB crossreacts immunologically with the B subunit of LT, the combined vaccine also shows some efficacy against ETEC.

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